1-Methylmalate from Camu-Camu (Myrciaria dubia) Suppressed D-Galactosamine-Induced Liver Injury in Rats

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To evaluate the protective effects of fruit juices against D-galactosamine (GalN)-induced liver injury, lyophilized fruit juices (total 12 kinds) were fed to rats for 7 d, and then we evoked liver injury by injecting GalN. The juice of camu-camu (Myrciaria dubia) significantly suppressed GalN-induced liver injury when the magnitude of liver injury was assessed by plasma alanine aminotransferase and aspartate aminotransferase activities, although some other juices (acerola, dragon fruit, shekwasha, and star fruit) also tended to have suppressive effects. An active compound was isolated from camu-camu juice by solvent fractionation and silica gel column chromatography. The structure was determined to be 1-methylmalate. On the other hand, malate, 1,4-dimethylmalate, citrate, and tartrate had no significant effect on GalN-induced liver injury. It is suggested that 1-methylmalate might be a rather specific compound among organic acids and their derivatives in fruit juices in suppressing GalN-induced liver injury.

Key words: D-galactosamine; liver injury; camu-camu; Myrciaria dubia; 1-methylmalate

Nutritional guidelines recommend one to intake fruits and vegetables at relatively high levels so as to prevent disease and maintain a healthy condition.1) Juices are convenient means of taking nutrients and other constituents of fruits and vegetables. Technological improvement of processing, transportation, and conservation have enabled juices of fruits which have not so far been utilized in Japan to appear on the market, but the nutritional and physiological effects of novel fruit juices have not yet been fully elucidated. It is generally thought that fruits and vegetables are nutrient sources of vitamins, minerals, and dietary fibers and thereby have nutritional effects. We have demonstrated that several kinds of fruits have suppressive effects on D-galactosamine (GalN)-induced liver injury in rats when various kinds of lyophilized fruits were added to the diet, suggesting that certain fruits have hepatoprotective effects.2) In the present study, we conducted a screening test for the liver injury-suppressive effects of 12 kinds of fruit juice, mainly of novel fruit juices, using rats intoxicated with GalN to assess the physiological effect of each fruit juice. Since camu-camu (Myrciaria dubia) juice had a potent suppressive effect, we attempted to isolate active compounds from the fruit juice.

Materials and Methods

Materials. Market products of 12 kinds of fruit juice concentrate were used. Acerola (Malpighia glabra), blackcurrant (Ribes nigrum), and blueberry (Vaccinium corymbosum) were purchased from SVZ International (Etten-Leur, Netherlands). Camu-camu (Myrciaria dubia) was from Amazon Camu Camu (Osaka, Japan). Litchi (Litchi chinensis), passion fruit (Passiflora edulis), and pomegranate (Panica granatum) were from Japan SiberHegner (Tokyo). Cranberry (Vaccinium oxyccoccus) and sea buckthorn (Hipppophae rhamnoides) were from Daishi Techno-Research (Tokyo). Dragon fruit (Hylocereus costaricensis) and star fruit (Averrhoa carambola) were from Oyama (Kobe, Japan). Shekwasha (Citrus depressa) was from Taketombo (Yokohama, Japan). Silica gel was from Sigma Aldrich (St. Louis, MO). Silica gel (Kieselgel 60) for column chromatography and silica gel TLC plates (Kieselgel 60 F254) were from Merck (Darmstadt, Germany), and other chemical reagents and solvents were from Wako Pure Chemical Industries (Osaka, Japan). Casein was from Nacalai Tesque (Kyoto, Japan). Mineral and vitamin mixtures (AIN-93) and cellulose powder were from Oriental Yeast (Tokyo), and the other ingredients of the diet were from Wako Pure Chemical Industries.

General procedures. 1H-NMR spectra (one-dimensional) were recorded on a JEOL lambda-500 spectrometer at 500 MHz, and 13C-NMR spectra were recorded on the same instrument at 125 MHz. ESIMS spectra were measured on a JMS-T100LC mass spectrometer. The final separation of compounds from camu-camu by HPLC was performed with a JASCO Galliliver system using a preparative column (Capcellpak C18 AQ; Shiseido, Tokyo).

Extraction and isolation of active compounds from camu-camu. Liquid camu-camu juice concentrate was successively extracted with ethyl acetate (EtOAc), n-butanol, and 70% ethanol, giving 4 fractions (frations 1–IV). Fraction I was applied to a silica gel column (8 x 60 cm) and eluted successively with n-hexane/EtOAc (8:2, 7:3, 5:5, and 0:10), EtOAc/acetone (5:5 and 0:10), and methanol, giving 5 fractions (fractions 1-1–1-5). Fraction 1-2 was further separated by silica gel column chromatography with a small open column (3 x 30 cm), and two major compounds (1 and 2) were obtained.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GalN, D-galactosamine; LDH, lactate dehydrogenase
These two compounds were finally purified by preparative HPLC (column, Capcellpak C18 AQ, 20 × 250 mm; solvent, 50% methanol; flow rate, 5 ml/min; detection, 239 nm). Compound 1 was the same as a major constituent of fraction I-1, and compound 2 was a major constituent of fraction I-2.

Animals and diets. In this study, six separate animal experiments were conducted to assess liver injury-suppressing effects. Six-week-old male conventional rats of the Wistar strain (120–130 g) were purchased from Japan SLC (Hamamatsu, Japan). The rats were individually housed in hanging stainless steel wire cages kept in a room at controlled temperature (23–25°C) and humidity (50–60%). Lights were maintained on a 12 h light-dark cycle (lights on from 7:00 to 19:00). The rats were acclimated to the facility for 4–5 d and given free access to water and the control diet. The composition of the control diet was as follows (%): casein, 25; cornstarch, 43.25; sucrose, 20; corn oil, 5; mineral mixture (AIN-93G), 3.5; vitamin mixture (AIN-93), 1; choline bitartrate, 0.25%; and cellulose, 2. Lyophilized fruit juices were added to the diet at the expense of cornstarch, since the dried juices contained relatively high levels of sugars (86–96% by dry weight basis; data from suppliers). Lyophilized camu-camu juice contained sugars at a level of 95.5%. After adaptation to the control diet, the rats were divided into groups and allowed free access to water and the experimental diets for 7 d.

In experiment 1, the rats were fed the control diet or diets supplemented with a powder of fruit juices at a level of 10%. The various fruit juices were lyophilized and powdered with a mixer and added to the control diet. In experiment 2, the rats were fed the control diet or diets supplemented with the various fractions derived by solvent extraction (fractions I–IV) of camu-camu at levels comparable to the addition of lyophilized camu-camu juice at the 10% level (fraction I, 0.87%; fraction II, 6.0%; fraction III, 2.6%; fraction IV, 0.59%). In experiment 3, the rats were fed the control diet or diets supplemented with the various fractions derived by silica gel column chromatography (fractions I-1–I-5) at levels comparable to the addition of fraction I (fraction I-1, 0.054%; fraction I-2, 0.18%; fraction I-3, 0.34%; fraction I-4, 0.042%; fraction I-5, 0.24%). In experiments 4–6, the rats were fed the control diet, and test samples were force-fed singly by stomach tube 4 h before injection of GaIN. In experiment 4, compounds 1 and 2 suspended in 0.5% methylcellulose solution were administered at a level of 500 mg/kg of body weight. The rats of the normal and control groups received vehicle only. In experiment 5, compound 2 was administered at levels of 125, 250, 500, and 1000 mg/kg of body weight. In experiment 6, malate, citrate, and tartrate were administered at levels of 125, 250, 500, and 1000 mg/kg of body weight. In experiment 6, malate, citrate, and tartrate were administered at a level of 500 mg/kg of body weight. After 7 d of feeding of the experimental diets (experiments 1–3) or after 4 h of administration of the test samples (experiments 4–6), GaIN was injected intraperitoneally at a dose of 350 mg/kg of body weight in accordance with our previous study.2 Test samples were administered 4 h before injection of GaIN, since a preliminary experiment showed that the effect of fraction I was stronger in rats administered it 4 h earlier than in rats administered it 2 h earlier. Untreated normal rats were injected with saline. At 24 h after injection of GaIN, the rats were killed by decapitation to obtain blood. The rats were not starved either before or after the injection of saline or GaIN. The study was approved by the Animal Use Committee of Shizuoka University, and the animals were maintained in accordance with the “Guidelines for the Care and Use of Laboratory Animals” of Shizuoka University.

Biochemical analysis. To assess the magnitude of liver injury, the activities of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured with a kit, Transaminase C II-Test (Wako Pure Chemical Industries). In experiment 4, plasma lactate dehydrogenase activity and the plasma bilirubin concentration were also measured with kits, LDH-Kainos (Kainos Laboratories, Tokyo) and the Bilirubin Assay Kit QuantiChrom (BioAssay Systems, Hayward, CA), respectively. Enzyme activities were expressed as mmol/min/l at 25°C.

Statistical analysis. Data values are expressed as mean±SEM. Data were analyzed by one-way ANOVA, and differences among experimental groups were analyzed by the Scheffe test when the F value was significant. Only the significance for the control group is expressed in the figures. A p value of 0.05 or less was considered significant. Statistical analysis was performed using Mac Toukei-Kaisekiver 1.5 software (Esumi, Tokyo).

Results

Effects of various fruit juices on d-galactosamine-induced liver injury (experiment 1)

The growth and food intake of rats during the 7 d experimental period were not affected by the addition of a powder of lyophilized fruit juice at a level of 10% (data not shown). Plasma ALT and AST activities were markedly increased by the injection of GaIN as compared with the normal rats (Fig. 1, panels A and B). The GaIN-induced increases in these enzyme activities were significantly suppressed in the rats fed camu-camu juice. Some other juices (acerola, dragon fruit, shekwasha, and star fruit) also tended to have suppressive effects, although there was no statistical significance. There was a significant correlation between
ALT activity and AST activity among the mean values of the 14 groups ($r = 0.986$, $p < 0.01$).

**Bioassay-guided fractionation of camu-camu (experiments 2 and 3)**

Liquid camu-camu juice concentrate, which was concentrated to one-half its initial volume, was extracted with ethyl acetate (fraction I), and the residue was further extracted with n-butanol (fraction II). The residue was divided into a 70% ethanol-soluble fraction (fraction III) and an insoluble fraction (fraction IV) by adding a 2.5-fold volume of ethanol. The yield of each fraction is shown in Fig. 2. GalN-induced enhancement of plasma ALT activity was significantly suppressed by fraction I when fractions I–IV were added to the control diet at levels comparable to the addition of lyophilized camu-camu juice at a level of 10% (Fig. 3, panel A). AST activity was significantly suppressed by fractions I and III (Fig. 3, panel B).

Fraction I was separated by column chromatography on a silica gel to give 5 fractions (fractions I-1–I-5), since the liver injury-suppressing activity of fraction I tended to be slightly greater than the activity of fraction III. The yield of each fraction is shown in Fig. 4. The GalN-induced increases in plasma ALT and AST activities were significantly suppressed only by fraction I-2 when the 5 fractions were added to the control diet at levels comparable to the addition of lyophilized camu-camu juice at a level of 10% (Fig. 5, panels A and B).

**Isolation of the liver injury-suppressing compound from camu-camu (experiments 4 and 5)**

Fraction I-2 was subjected to separation of constituents by silica gel column chromatography, followed by purification by preparative HPLC, since only fraction I-2 had liver injury-suppressing activity. Two compounds, 1 and 2, were isolated from fraction I-2. Compound 2 was a major constituent of fraction I-2 and compound 1 was also in fraction I-1 as a major constituent. The yields of compounds 1 and 2 were 0.048% and 0.159% of lyophilized camu-camu juice respectively. The rats were force-fed singly compounds 1 and 2 at a dose of 500 mg/kg of body weight to assess the liver injury-suppressing activity of compounds 1 and 2.

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**Fig. 2.** Fractionation of Camu-Camu Juice Concentrate by Successive Extraction with Organic Solvents and the Yields of the Various Fractions.

**Fig. 3.** Effects of Dietary Addition of Fractions I to IV from Camu-Camu Juice on d-Galactosamine-Induced Increases in Plasma Alanine Aminotransferase (A) and Aspartate Aminotransferase (B) Activities in Rats (Experiment 2).

Each column and its bar represent the mean value and SEM respectively for 5 (normal group) or 10 rats (the other groups). **Significantly different from the control group at $p < 0.05$ and $p < 0.01$ respectively.

**Fig. 4.** Fractionation of Fraction I from Camu-Camu Juice by Silica Gel Column Chromatography and the Yields of the Various Fractions.

**Fig. 5.** Effects of Dietary Addition of Fractions I-1 to I-5 from Camu-Camu Juice on d-Galactosamine-Induced Increases in Plasma Alanine Aminotransferase (A) and Aspartate Aminotransferase (B) Activities in Rats (Experiment 3).

Each column and its bar represent the mean value and SEM respectively for 5 (normal group) or 10 rats (the other groups). **Significantly different from the control group at $p < 0.05$ and $p < 0.01$ respectively.
suppressing activity, since the obtained amounts of these compounds were limited. GalN-induced increases in plasma ALT, AST, and LDH activities were significantly suppressed by compound 2, but not by compound 1 (Fig. 6, panels A, B, and C). The GalN-induced increase in plasma bilirubin concentration was also significantly suppressed by compound 2, but not by compound 1 (Fig. 6, panel D).

Each column and its bar represent the mean value and SEM respectively for 5 (normal group) or 10 rats (the other groups). **Significantly different from the control group at \( p < 0.05 \) and \( p < 0.01 \) respectively.

A single force-feeding of 1-methylmalate at levels of 125, 250, 500, and 1,000 mg/kg of body weight dose-dependently prohibited GalN-induced increases in plasma ALT and AST activities, although the effect of the compound was not significant at a dose of 125 mg/kg (Fig. 8, panels A and B).

**Effects of general organic acids on GalN-induced liver injury (experiment 6)**

The effects of the general organic acids in fruit juices on GalN-induced liver injury were investigated to determine whether the effect of 1-methylmalate is specific. A single force-feeding of malate, citrate, and tartrate at a level of 500 mg/kg of body weight did not suppress GalN-induced elevations of plasma ALT or AST activities (Fig. 9, panels A and B).

**Discussion**

Various drugs have been used to induce experimental liver injury. GalN is one of these drugs, and it has often been used to assess hepatoprotective foods, medicines, and their constituents. For instance, recent reports have shown the suppressive effects of seaweed (Ulva lactuca), mushroom (Ganoderma lacidum), and...
medicinal plant (Sedum sarmentosum), and an herb (Cajanus indicus) on GaIN-induced liver injury in rodents and hepatocytes. We have also found that GaIN-induced liver injury was effectively suppressed by green tea, mushrooms, fruits, and dietary fibers in rats. The present study indicates that some fruit juices (acerola, camu-camu, dragon fruit, shekawasha, and star fruit) possess or tend to possess suppressive effects on GaIN-induced increases in plasma ALT and AST activities, representative markers for the magnitude of liver injury, when lyophilized fruit juices were added to the diet at a 10% level. These results suggest that these fruit juices suppress or tend to suppress certain types of liver injury, such as that induced by GaIN.

We have isolated 5 fatty acid derivatives from avocado (Persea americana) as GaIN-induced liver injury-suppressive compounds. In the present study, we isolated 1-methylmalate from camu-camu juice, and this had the most potent suppressive effect on GaIN-induced liver injury as an active compound. In general, fruits contain several organic acids at relatively high levels. However, it appears that the liver injury-suppressive effect of 1-methylmalate might be rather specific among the organic acids and methyl derivatives in fruits, since organic acids (malate, citrate, and tartrate) and 1,4-dimethylmalate had no significant effect. Furthermore, we measured plasma LDH activity and bilirubin concentration in addition to ALT and AST activities as liver injury markers to confirm the effect of 1-methylmalate. That 1-methylmalate also suppressed GaIN-induced increases in plasma LDH activity and the plasma bilirubin concentration indicates that the suppressive effect of 1-methylmalate on GaIN-induced liver injury was not artificial, e.g., not due to the inhibition of plasma transaminases (ALT and AST) by the compound.

It is thought that GaIN induces liver injury by inhibiting the synthesis of RNA and proteins through a decrease in the hepatic UTP concentration, which finally evokes the necrosis of liver cells. On the other hand, recent reports have shown that several cytokines, e.g., interleukin (IL)-1α, IL-6, and IL-18, and NO might be associated with the pathogenesis of GaIN-induced liver injury, but the mechanism by which 1-methylmalate suppresses GaIN-induced liver injury remains unclear.

There are several reports on the existence and biological activities of 1-methylmalate. Han et al. have reported that the 1-methylmalate found in the fruits of a species of cactus (Opuntia ficus-indica var. saboten), exhibited a monoamine oxidase-inhibitory activity. Furthermore, Suga et al. have found that the 1-methylmalate found in the fruits of a medicinal plant (Myrica rubra) inhibited the lipopolysaccharide-induced production of IL-10 and augmented the lipopolysaccharide-induced production of IL-12 and tumor necrosis factor-α in human peripheral blood mononuclear cells. No other biological activity of 1-methylmalate has not been demonstrated to our knowledge, although some reports have shown that the compound also exists in several plants, e.g., Rheum maximowiczii, Agave Americana, and Vaccinium macrocarpon. We report here for the first time that 1-methylmalate has hepatoprotective activity.

Camu-camu is a fruit originating in Peru that contains a high level of vitamin C. Recently, Inoue et al. reported that camu-camu suppressed several oxidative and inflammatory markers in humans when smoking male volunteers took 70 ml of 100% camu-camu juice for 7 d, but tablets containing a corresponding amount of vitamin C did not exhibit any significant effect, indicating that camu-camu juice has anti-oxidative and anti-inflammatory effects in humans under an accelerated oxidative stress state. They concluded that the effects of camu-camu juice might be due to the existence of unknown anti-oxidant substances besides vitamin C, or to unknown substances modulating in vivo vitamin C kinetics. It is uncertain whether 1-methylmalate participates in the anti-oxidative and anti-inflammatory effects reported by them. In humans, liver injury (hepatitis) is caused by viruses, chemicals, alcohol, autoimmune diseases, etc. Hence, it is interesting to know whether some fruit juices, which have suppressive effects on GaIN-induced liver injury, also have suppressive effects on other types of liver injury, e.g., virus-induced hepatitis. This remains to be elucidated.

References